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NUTRITION REVIEWS

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NUTRITION REVIEWS

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THE COMPARATIVE VALUE OF NIACIN AND NICOTINAMIDE

In 1937 C. A. Elvehjem, R. J. Madden, F. M. Strong, and D. W. Woolley (*J. Am. Chem. Soc.* **59,** *1767 (1937); J. Biol. Chem.* **123,** *137 (1938)*) showed that nicotinamide, separated from liver, and free nicotinic acid were effective in the cure of blacktongue in dogs. The successful treatment of human pellagra with nicotinic acid was reported by P. J. Fouts, O. M. Helmer, S. Lepkovsky, and T. H. Jukes (*Proc. Soc. Exp. Biol. Med.* **37,** *405 (1937)*), and by T. D. Spies, C. Cooper, and M. A. Blankenhorn (*J. Am. Med. Assn.* **110,** *622 (1938)*).

It is believed that nicotinamide occurs free in the tissues and also in combined form in a number of enzyme systems such as the coenzymes I and II, but to a much greater extent in the bound form than as the free amide. These enzymes are involved in a wide variety of reactions and are of great importance in animal physiology as well as plant metabolism.

Free nicotinic acid apparently does not exist in the living organism but it is found in the urine of animals (H. R. Rosenberg, "Chemistry and Physiology of the Vitamins," *Interscience Publishers, Inc., New York, N. Y. (1942)*). It can, however, be isolated from natural sources after the material has been saponified. On acidification, free nicotinic acid can be recovered. This brief discussion of how nicotinic acid occurs in living matter is to place emphasis upon the important point that it is the amide and not the free acid that is the principal form in living tissues.

After the relation of nicotinic acid and nicotinamide to the treatment of pellagra had been disclosed, it was natural for investigators of this problem to study the comparative effectiveness of a variety of compounds with somewhat similar structure, but all containing the pyridine nucleus. Other closely related structures such as quinolinic acid, pyrazine 2–3-dicarboxylic acid and pyrazine monocarboxylic acid were investigated but it is definitely established at the present time that these compounds are greatly inferior to nicotinamide or nicotinic acid in the treatment of pellagra or in counteracting nicotinamide deficiency.

Out of these experiences with a variety of pyridine derivatives for the treatment of pellagra or the reinforcement of food there have survived the two compounds, nicotinamide and nicotinic acid (niacin). The latter, because it was derived from tobacco by the oxidation of nicotine and also because it was an acid suffered early objections, and finally was christened niacin to remove the stigma of origin and structure.

As more experience with niacin has accumulated, further objections to its use have appeared both in the clinic and in the food field. It should, however, be stated that up to the present time (January, 1946) the cost of niacin as compared with the nicotinamide has been favorable to the former, although cost differences are gradually decreasing. At present the difference in price is of no real significance.

The clinician's choice of nicotinamide over nicotinic acid in the treatment of subclinical and classic pellagra is warranted because of the unpleasant reactions induced by nicotinic acid therapy and further by the fact that the two compounds have equal therapeutic effectiveness.

The nitritoid reactions, similar to the effects of histamine, may occur in man with as little as 30 mg. of nicotinic acid per day, orally, as was shown in a careful study by W. H. Sebrell and R. E. Butler (*J. Am. Med. Assn.* **111,** *2286 (1938)*).

D. T. Smith, J. M. Ruffin, and S. G. Smith (*J. Am. Med. Assn.* **109,** *2054 (1937)*)

observed a marked flushing of the face, neck, and arms a few minutes after the intramuscular injection of 60 mg. of nicotinic acid and with as little as 12 mg. administered intravenously.

T. D. Spies, W. B. Bean, and R. E. Stone (*J. Am. Med. Assn.* **111,** *584 (1938)*) report that the flushing reaction can be produced in all persons, although the amount required to produce it is extremely variable at different times in the same person and from one person to another. This extreme variability is also the experience of many other observers and is probably related at least in part to the variable rate of absorption of this vitamin, since Spies *et al.* (*loc. cit.*) report that as little as 10 mg. of nicotinic acid intravenously produces a flushing reaction within one minute.

R. J. Popkin (*Am. Heart J.* **18,** *697 (1939)*) states that orally administered nicotinic acid in single doses of 30 to 120 mg. causes transitory flushing of the skin in a majority of cases regardless of the condition of the subject or the size of dose used and suggests that the effects of nicotinic acid are similar to those produced by histamine.

H. Field and W. D. Robinson (*Am. J. Med. Sci.* **199,** *275 (1940)*) gave oral doses of nicotinamide up to 500 mg. under fasting conditions and did not observe any untoward reactions such as were observed with nicotinic acid.

A. C. Alport and P. Ghalioungui (*Arch. f. Schiffs- und Tropen-hygiene* **43,** *34 (1939)*, abstracted in *Brit. Med. J.* **1,** *77 (1939)*) used nicotinamide in the treatment of pellagrins in Egypt with daily doses of 1 g. orally or 0.3 g. parenterally and found that "toxic" reactions were less severe than with nicotinic acid.

D. S. Robertson (*Brit. Med. J.* **1,** *339 (1941)*) in England observed that the amide is better tolerated than the acid and has indicated that it is possibly more effective in treatment.

Whether the flushing reaction induced by nicotinic acid is more profound in children than in adults cannot be clearly ascertained from the available literature. It should be understood, however, that the reactions observed with nicotinic acid are not to be construed as frank toxic manifestations, at least so far as is known.

In the food reinforcement field we have been unable to find any recorded literature assessing the comparative value of the two compounds. Personal communications have varied in their position as to any untoward effects from the use of niacin. However, these communications are agreed that when more than 10 mg. per day are ingested, the nicotinamide is preferable.

But in the food field where 16 mg. per pound of flour are added as a minimum—20 mg. as a maximum—and the present laws enacted in sixteen states require 16 mg. as a minimum, in general no objection to niacin has been definitely recorded. The consensus among cereal manufacturers is that for the cereals the acid (niacin) can be used not because it is better, but because it is cheaper.

As stated in the earlier part of this paper, the differential in cost is slight, but of course a factor. On the other hand, experience in the food field is too limited for a definite and final claim as to the equality of the acid to the amide. There is great variability in the susceptibility of individuals to ingested chemical entities. With the recognition that such susceptibility exists, the compound known to have deleterious effects should be avoided and the one proved to be innocuous should be selected, even in the food field.

We have received a report of a patient who would flush with the amount of niacin contained in 60 g. of enriched white flour (about 2 mg.), while other persons could tolerate as much as 100 mg. of the acid without apparent effect. Thus it would appear desirable to depend on the nicotinamide for clinical use and for fortification of foods.

Recently it was reported to us that one large cereal company had changed from the acid compound (niacin) to the nicotinamide

because some of the operators in their plant were affected by niacin and because it was desired to take advantage of the innocuous clinical properties of nicotinamide. This change was made despite a slight cost differential in favor of niacin. This we believe to be a move in the proper direction, especially when the type of consumer is as varied as in the food field.

The use of nicotinamide in place of nicotinic acid in food fortification should not cause technologic difficulty since both compounds have the following properties in common: (a) solubility (the amide is more soluble), (b) biologic activity, and (c) ease of measurement.

In the field of human and animal nutrition when definite chemical substances are competing for use, it should be clear that when the price difference is not too great, the chemical entity possessing equally effective curative or supplemental properties and possessing the least objectionable properties should be the compound of choice. In the case under discussion this choice should be nicotinamide. Freedom from objectionable aftereffects, wherever possible, should be the objective of clinicians using new chemical compounds for curing or preventing disease, —and quality products, irrespective of slight cost advantages, should also be the objective of food manufacturers.

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PRESENT KNOWLEDGE OF VITAMIN A IN NUTRITION

Vitamin A is an unsaturated cyclic alcohol having the empirical formula $C_{19}H_{27}CH_2OH$. Its synthesis has been reported twice but both claims lack confirmation, although its relationship to beta-carotene leaves little doubt of its structure. It is found only in the animal kingdom, where it occurs presumably in all species of fish, birds, and mammals. Ordinarily the chief store is found in the liver, but when larger quantities collect in the body, the kidneys, lungs, and even fat depots may contain considerable amounts. Some of the vitamin A is present in the form of the free alcohol, but the greater part of it occurs as esters of the higher fatty acids and related unsaturated acids. Probably for each species there is a characteristic mixture of vitamin A esters; when a different ester from that which ordinarily predominates in the liver of a species is fed, the vitamin A ester mixture found in the liver some time later approximates that ordinarily found.

The source of all the vitamin A in the animal kingdom, so far as is known, is in the carotenes and certain closely related carotenoids which are synthesized by all plants except parasites and saprophytes. Fish make vitamin A from the carotenoids of the marine plankton, and herbivorous land animals from the carotenoids of the green leafy foods on which they graze. Carnivorous animals are dependent for their supply upon the vitamin A already transformed from carotenoids by the herbivores.

Both vitamin A alcohol and some of its esters have been obtained in crystalline form. Vitamin A itself is a pale yellow substance showing a characteristic absorption spectrum with a band having maximum absorption at 325 to 328 mμ. It is soluble in fat solvents. In chloroform solution it reacts with antimony trichloride to produce an intensely blue compound with an absorption band in its spectrum at 620 mμ. Each of these properties has been made the basis of methods for physico-chemical assay of the vitamin. Neither type of assay is wholly satisfactory, since breakdown products and other closely related compounds also absorb

ultraviolet radiation at 3280 Å and give rise with antimony trichloride to blue compounds which absorb light of wavelength 6200 Å.

In addition to the vitamin A long known to occur in sea fish oils, another form named vitamin A_2 has been found in the oils of some fresh water fish. Vitamin A_2 has not been crystallized, but it possesses an ultraviolet absorption spectrum with maximum absorption at 345 to 350 mμ instead of at 325 to 328 mμ, and with antimony trichloride it yields a blue compound having an absorption maximum at 697 mμ instead of 620 mμ. Vitamin A_2 has been reported to be much less active in promoting the growth of rats than vitamin A_1 (i.e. the vitamin A of most studies), the ratio of the activities of vitamin A_2 to vitamin A_1 being possibly of the order of 1 to 100.

The carotenes and carotenoids which act as provitamins A are dark red crystalline compounds of high melting point which give yellow, orange, and red solutions in fat solvents as the concentration increases. Beta-carotene ($C_{40}H_{56}$) is symmetrical about the center of the carbon chain linking its two rings, and the molecule is broken down in the liver to give two molecules of vitamin A; the other provitamins are not symmetrical in the same way, and one molecule of each gives rise only to one molecule of vitamin A.

Provitamins A are not ordinarily found in large quantity in the healthy animal since they are transformed into vitamin A and stored or utilized in that form. Carotenoids which are not provitamins may be absorbed from the food and deposited with the body fats. It is believed that the transformation of provitamins occurs only in the liver. In general the provitamins of the diet are less well absorbed from the food than is vitamin A, and are more prone to suffer oxidative destruction in the intestinal tract.

One chief function of vitamin A in the body is the maintenance of epithelial tissues in a normal condition; the way in which this function is exercised remains uncertain. Another function is to participate in the so-called "visual cycle," the series of events by which dim vision is mediated in the rods of the retina. The visual pigment in the retinal rods of a number of species is rhodopsin (visual purple). The incidence of light on the rods causes very rapid breakdown of rhodopsin to visual yellow, which is a mixture of "retinene" and a protein. This mixture undergoes two slow reactions: (1) the re-formation of rhodopsin, (2) the transformation to visual white, which is a mixture of vitamin A and protein. Vitamin A and the protein can also be built into rhodopsin. During the continuous reactions occurring in the rods some part of the vitamin A or retinene slowly disappears; therefore unless more vitamin A is supplied by the blood an impairment of rod vision will occur which will give rise to a form of night blindness named "functional hemeralopia." A second closely similar pigment named porphyropsin is found in some species, entering into a visual cycle involving vitamin A_2 and retinene$_2$. The two pigments may occur separately or together in different species. Retinene yields an antimony chloride band at 664 mμ; it has been suggested that it is vitamin A aldehyde ($C_{19}H_{27}CHO$).

Apart from the hemeralopia of vitamin A deficiency most of the other pathologic changes follow upon a single cytologic change, which affects the mucous membranes throughout the body. These epitheliums atrophy and their basal cells proliferate, giving rise to keratinizing epithelium comparable with epidermis. The keratinized epitheliums are open to bacterial invasion, consequently severe infections of the eye (xerophthalmia), respiratory organs, genitourinary tract, and mouth have been observed in severe vitamin A deficiency. It has been stated that this deficiency is the cause of much blindness among the populations of the Orient. In young mammals the skeletal growth is retarded by vitamin A deficiency before the growth

of the soft tissues is affected. The continuing growth of the central nervous system within its bony framework then causes overcrowding of the cranium and spinal canal, thus leading to mechanical damage of the brain and spinal cord.

All green leafy vegetables, and yellow vegetables and fruits such as sweet potatoes and peaches supply provitamins A in the human diet. Preformed vitamin A is supplied by milk fat, liver, and to a lesser extent by kidney and the fat of muscle meats. The Food and Nutrition Board's Recommended Daily Allowance of vitamin A is 5000 I. U. for adults, which probably allows an ample margin of safety. Little experimental evidence is yet available on which to base a figure for actual requirement. Although some claims have been made that slight or moderate vitamin A deficiency is common in this country, it is probable that it is relatively rare, since the signs on which such claims have been based are not sufficiently specific. In the absence of clear clinical signs the only satisfactory evidence of vitamin A deficiency is probably the occurrence of subnormal dark adaptation which rapidly returns to normal after treatment with vitamin A. Levels of blood plasma vitamin A as low as those found in clinical vitamin A deficiency disease in China are rarely reported in this country. Low levels have been observed in a variety of diseases, particularly in febrile conditions and in some liver diseases. A high plasma carotene content concurrent with a low plasma vitamin A may be an indication of failure in liver function.

PANTOTHENIC ACID METABOLISM

Pantothenic acid was discovered as a result of its activity as a growth factor for some strains of yeast, and many other microorganisms require it for growth. D. W. Woolley (*J. Biol. Chem.* **130,** *417 (1939)*) observed that for the hemolytic streptococcus strain H 69 D pantothenic acid can be replaced by the lactone which can be isolated from the products of hydrolysis of pantothenic acid. L. A. Underkofler, A. C. Bantz, and W. H. Peterson (*J. Bact.* **45,** *183 (1943)*) found that the same is true for the nonpathogenic organism Acetobacter suboxydans. The other product of pantothenic acid hydrolysis, beta-alanine, is not essential for the growth of this species.

H. P. Sarett and V. H. Cheldelin (*J. Biol. Chem.* **159,** *311 (1945)*) have used this organism in devising a microbiologic assay for the estimation of the lactone. They observed that the dihydroxy-dimethyl-butyric acid which is obtained in the form of its salt on alkaline hydrolysis of the lactone or of pantothenic acid is four to five times as active in promoting the growth of A. suboxydans. Greater sensitivity was therefore obtained in their method by subjecting both the standard and the unknown solutions to alkaline hydrolysis, thus converting the lactone to the dihydroxy acid before assay. The response is measured by determining the turbidity of the bacterial suspension in a photocolorimeter after incubation at 30°C. for sixty-five hours, and the presence of 0.1 to 0.5 microgram of the lactone per assay flask gives results in the best range for assay work.

The method was not found suitable for assaying directly the lactone in the presence of considerable quantities of pantothenic acid; but in a solution containing both, hydrolysis with alkali under specified conditions converted both pantothenic acid and the lactone into the dihydroxy acid, and subsequent assay determined the "total lactone" present. By assaying the solution for pantothenic acid with Lactobacillus arabinosus by the method of E. H. Hoag, H. P. Sarett, and V. H. Cheldelin (*Ind. Eng. Chem.* (*Anal. Ed.*) **17,** *60 (1945)*), the

"pantothenic acid lactone" could be determined, and the difference between the two determinations represented the free lactone present.

These methods for the determination of pantothenic acid and of its lactone portion have been applied by H. P. Sarett (*J. Biol. Chem.* **159,** *321 (1945)*) in an attempt to follow the pathway of pantothenic metabolism in the human being. The work of P. B. Pearson (*Am. J. Physiol.* **135,** *69 (1941)*) and of R. A. McLean, D. T. Smith, and S. G. Smith (*Abstracts, Am. Chem. Soc., 108th Meeting, New York City (1944)*) had already shown that only a small part of a large dose of pantothenic acid is excreted in the urine.

This finding was confirmed by Sarett for 3 subjects, and it was also established that none of the free lactone existed in normal urine (urine secreted on an ordinary diet, without pantothenic acid dosing). The observed daily excretion of pantothenic acid itself as measured by the L. arabinosus assay was on the average 3.5 mg.; the range was from 1.3 to 5.3 mg. After an oral dose of 100 mg. of pantothenic acid, the subjects excreted from 8.8 to 23.4 mg. of pantothenic acid above the basal excretion level, but the urines also contained from 0.6 to 6.1 mg. of free lactone in addition to the pantothenic acid. When the 100 mg. dose of pantothenic acid was given intravenously, from 22 to 41 mg. appeared in the urine unchanged, and no free lactone was found.

To test whether the lactone could be metabolized further, 50 mg. doses were given both orally and intravenously. The lactone was recovered quantitatively from the urine; when it was given orally, but not when it was given intravenously, the average output of pantothenic acid increased from 3.5 mg. to 6.2 mg. per day. The alkali-hydrolyzed lactone (salt of the dihydroxy acid) also was excreted quantitatively when given intravenously, but when given orally, 2 of the subjects excreted only about 80 per cent of the dose while the third excreted 97 per cent.

It appears established that the lactone and dihydroxy acid of pantothenic acid are not normal intermediates in the metabolism of pantothenic acid by man.

BIOLOGIC ACTIVITY OF THIENYLALANINE

Much interest has accrued in recent years to the discovery that certain compounds related structurally to growth factors can inhibit the action of these essential substances. Most attention has been given to compounds related to the vitamins (*Nutrition Reviews* **2,** *28 (1944)*) but comparable studies of compounds related structurally to some of the amino acids have also been pursued. Thus H. M. Dyer (*J. Biol. Chem.* **124,** *519 (1938)*) compared the action of ethionine with that of the essential amino acid methionine, the two compounds being related as successive members of a homologous series. It was observed that ethionine was toxic for young rats maintained on a diet free of methionine, and that the toxicity could be prevented by the addition of methionine to the diet. J. S. Harris and H. I. Kohn (*J. Pharmacol. Exp. Therap.* **73,** *383 (1941)*) later observed that the growth of Escherichia coli was inhibited by ethionine, but here too the inhibition was completely overcome by methionine.

R. O. Roblin *et al.* (*J. Am. Chem. Soc.* **67,** *290 (1945)*) have extended the study of compounds related to methionine to its oxygen analogue. This compound differs from methionine only in the replacement of its sulfur atom by an oxygen atom, and perhaps may therefore be regarded as more closely related to methionine since it is isosteric with methionine. Just as with ethionine, they observed that addition of

methoxinine (the oxygen analogue) to the medium inhibited the growth of E. coli, and that methionine reversed the inhibition. They also noted the specificity of the reaction, finding that the reversal could be brought about by l-methionine but not by d-methionine.

V. du Vigneaud *et al.* (*J. Biol. Chem.* **159,** *385 (1945)*) have now examined the action of beta-2-thienylalanine, a compound which is isosteric with phenylalanine, also an essential amino acid. Working with the yeast Saccharomyces cerivisiae strain 139 in a medium of known composition slightly modified from that described by E. E. Snell, R. E. Eakin, and R. J. Williams (*J. Am. Chem. Soc.* **62,** *175 (1940)*), growth was measured turbidimetrically after sixteen hours incubation at 30°C., using a photoelectric colorimeter. The effect of varying concentrations of thienylalanine was studied and it was determined that approximately 8 micrograms of thienylalanine per milliliter of medium reduced the growth rate on the basal medium by about 50 per cent. The addition of 57 micrograms of thienylalanine per milliliter of medium reduced the growth to about 17 per cent of that on the basal medium, and greater concentrations of thienylalanine caused no further inhibition.

With 57 micrograms of thienylalanine per milliliter, the inhibition could be reversed by the addition of phenylalanine. The growth rate returned to 50 per cent of that on the basal medium when about 50 micrograms of phenylalanine were added per milliliter of medium; it returned almost to the full normal rate on the basal medium when 120 micrograms of phenylalanine were added per milliliter of medium. Tyrosine in similar concentrations had no effect on the inhibitory action of thienylalanine.

In tests with young rats, a basal diet was first used which supplied all the essential amino acids except phenylalanine. After four days, during which they declined steadily in weight, phenylalanine was added to the diet of 2 of the rats, and thienylalanine to the diet of the other 2. It was evident within a few days that thienylalanine could not replace phenylalanine, since the rats which received it continued to decline in weight.

Since H. C. Yuan and H. C. Li (*J. Chinese Chem. Soc.* **5,** *214 (1937)*) had indicated a certain structural similarity between thienylalanine and methionine, a group of 4 rats was used to observe the effect of adding thienylalanine to a diet devoid of methionine. Here again it did not serve to reverse the growth failure, and so cannot replace methionine as a source of sulfur. No evidence was obtained which would suggest that thienylalanine exerts an "antiphenylalanine" effect in the rat, although the possibility cannot be ruled out without further investigation.

THIAMINE DEFICIENCY, INANITION, AND ANESTRUS

Nutrition investigations involving experimental animals are frequently carried out with an ad libitum feeding technic. This procedure is less laborious than paired feeding. When an animal is suffering from an uncomplicated single vitamin deficiency, for example, a great improvement of appetite and a marked increase in weight following therapy appears to be an almost infallible indication that the deficiency has been corrected. It is probable that the animal's utilization of food has also improved following therapy, and this might be shown by paired feeding with an untreated animal. One would not expect, however, to obtain as striking results as with ad libitum feeding. On the other hand, for other types of experiments control of food intake is essential. Signs which appear in animals with vitamin or other deficiencies may not be specific for the deficiency in question but may result from the accompanying inanition. To dif-

ferentiate between these possibilities, it is necessary to employ some version of the paired feeding procedure. Vitamin A was long considered as a more or less specific "anti-infective" factor but data obtained in controlled feeding experiments indicate that it is diminution of food intake rather than a deficiency of vitamin A which increases the susceptibility of animals to various infections (*Nutrition Reviews* **3,** *229 (1945)*). The feeding of a diet deficient in thiamine leads to failure of estrus, but paired feeding studies have shown that the development of this defect also is actually due to the accompanying inanition.

It was shown by H. M. Evans and K. S. Bishop (*J. Met. Res.* **1,** *335 (1922)*) that the absence of "vitamin B" resulted in a complete cessation of the estrus cycle. It was not known at this time that "vitamin B" was multiple in nature. K. H. Coward and B. G. E. Morgan (*Biochem. J.* **35,** *974 (1941)*) concluded from experiments which they performed that anestrus could result from a deficiency of either thiamine or riboflavin. They did not use pure vitamins in their experiments but were able to assay concentrates for their thiamine content with fair accuracy by using the recovery from anestrus as a test. In both of these studies the effect on the ovary could have been due either to the vitamin deficiencies *per se* or to the resulting inanition. Evans and Bishop noted in their report that underfeeding of rats with an otherwise complete diet resulted in aberration of the ovulation cycle. The procedure used restricted the intake of "vitamin B" as well as that of other ingredients of the diet.

M. G. Mulinos, L. Pomerantz, J. Smelser, and R. Kurzrok (*Proc. Soc. Exp. Biol. Med.* **40,** *79 (1939)*) showed that complete or partial starvation led to complete inhibition of the estrus cycle in rats and that this inhibition was roughly related to the loss in body weight. When the animals were fed again there was a return to the normal cyclic changes in the vaginal mucosa. Either estradiol monobenzoate or gonadotropic hormone injections were followed by estrus in the starved animals and Mulinos *et al.* concluded that inanition produces anestrus through changes induced in the hypophysis. They called attention to the fact that in the earlier experiments showing anestrus resulting from vitamin deficiencies (and from other causes), all experimental animals lost considerable weight. The authors advanced the hypothesis that in all of these experiments the failure of estrus was wholly or in part due to the concomitant inanition. In their own work, however, no data were given as to the vitamin intake of their animals, and no proof of their hypothesis was offered.

V. A. Drill and M. W. Burrill (*Endocrinology* **35,** *187 (1944)*) studied the relative importance of thiamine deficiency and inanition in producing anestrus, using a paired feeding technic. Adult female white rats were fed a purified diet and were given a supplement containing thiamine, riboflavin, pyridoxine, pantothenic acid, niacin, biotin, inositol, choline, and para-aminobenzoic acid. After ten days on this dietary regimen the experiment proper was begun. The rats were divided into two groups, those of one group (the controls) being continued on the same diet and supplement, while for the others the only change was omission of thiamine from the supplement. The thiamine deficient animals received food ad libitum but each control animal was allowed only as much food as the average amount consumed by the deficient animals. Thirteen of 15 deficient rats and 11 of 15 controls showed persistent anestrus after sixteen to twenty-nine days on experiment. A few animals in each group were later given subcutaneous injections of a gonadotropic substance. All such animals, whether thiamine deficient or inanition controls, responded by reaching a state of complete estrus. Drill and Burrill concluded that the effect of thiamine deficiency on the ovary is not one of thiamine deficiency *per se* but rather is due to the

resulting inanition. They concluded also, in agreement with previous investigators, that the continued diestrus resulting from inanition is due to failure of the pituitary to produce sufficient gonadotropic substance rather than to ovarian failure or to loss in sensitivity of the vaginal epithelium.

The data presented serve to emphasize once more that a sign observed in an animal suffering from a dietary deficiency cannot be considered to be specific for that deficiency unless it can be shown that animals serving as inanition controls fail to develop the sign in question.

DENTAL CARIES IN THE COTTON RAT

The subject of dental caries in the cotton rat was dealt with in this journal some months ago (*Nutrition Reviews* **3,** *83* (*1945*)). Since that time additional information has been presented in three papers from the Wisconsin laboratories. In the earlier work it was established that in the cotton rat a simplified ration high in sucrose caused a high incidence of dental caries, but a similar ration in which the sucrose was replaced completely by dextrin caused a much lower incidence of caries. When a stock ration was fed for a similar period, a still lower incidence of carious lesions was observed.

B. S. Schweigert, J. H. Shaw, P. H. Phillips, and C. A. Elvehjem (*J. Nutrition* **29,** *405* (*1945*)) studied the effect of partially or completely replacing the sucrose in the basal ration by other carbohydrates. When one half of the sucrose was replaced by dextrin, there was no evidence that the resulting incidence of caries was decreased; but when three quarters of the sucrose was replaced by dextrin, the incidence of carious lesions was reduced by about 30 per cent. When glucose, fructose, maltose, dextri-maltose, or a mixture of lactose and sucrose was used, the incidence of tooth decay remained high. With the small number of cotton rats in each group, no evidence of differences was observed in the caries incidence resulting from the use of these different sugars in the diet. However it was clearly established that in the cotton rat the use of 67 per cent of these soluble sugars in the diet favored the formation of carious lesions, while dextrin did not favor them.

J. H. Shaw, B. S. Schweigert, P. H. Phillips, and C. A. Elvehjem (*Proc. Soc. Exp. Biol. Med.* **59,** *89* (*1945*)) reported the effect of sodium fluoride additions to their basal ration upon the incidence of carious lesions in the cotton rat. These supplements were begun at weaning and continued through the normal fourteen week experimental period. The crowns of the first and second molars were fully formed and the crowns of the third molars were partially formed when the fluorine supplements were begun. It was found that there was no inhibitory effect on caries development when 22.5 parts per million of fluorine were added to the basal ration. The addition of 45, 90, or 135 p.p.m. of fluorine caused a partial inhibition of caries formation. When 225 or 450 p.p.m. of fluorine were ingested, a 90 per cent decrease in caries incidence resulted. Depigmentation of the incisors occurred when 45 or more p.p.m. of fluorine were ingested, while mottling of the enamel of the third molars occurred when 225 or more p.p.m. were fed. It is interesting to note the high amounts of fluorine required to inhibit caries development in the cotton rat. Similar amounts of fluorine have been required to produce an 80 to 85 per cent reduction of coarse particle caries in the white rat (H. C. Hodge and S. B. Finn, *Proc. Soc. Exp. Biol. Med.* **42,** *318* (*1939*); V. D. Cheyne, *Ibid.* **43,** *58* (*1940*)). Since the fluorine supplements in

the cotton rat experiments were given after the period of tooth formation was almost complete, it would be interesting to know if as large amounts had to be administered during tooth development. Fluorine analyses of the dextrin and sucrose used in earlier experiments (J. H. Shaw and co-workers, *J. Nutrition* **28,** *333 (1944)*) indicated that the protective effect of dextrin could not be explained by its fluorine content which was far below the level required to produce a comparable inhibition of caries formation.

A highly significant difference in the caries susceptibility of various strains of cotton rats was observed by B. S. Schweigert, J. H. Shaw, C. A. Elvehjem, and P. H. Phillips (*Proc. Soc. Exp. Biol. Med.* **59,** *44 (1945)*). The data presented and statistically analyzed in this report were collected from all previous experiments where the effect of stock diets and the diet containing dextrin was compared with the effect of diets containing soluble sugars. As an example of the difference in caries susceptibility of different strains of cotton rats, the average number of carious lesions for 18 siblings of a susceptible strain was 31.3 with a range of 22 to 37 in comparison with an average incidence of 13.0 and range of 5 to 23 for 7 siblings of a resistant strain. The various susceptible and resistant strains reacted similarly to dietary alterations except that the members of the resistant strains had a consistently lower caries incidence. The wide differences in susceptibility and the range of susceptibility within any strain make it imperative that the utmost care be used in selecting experimental animals and in interpreting the results obtained.

These three phases of dental caries research in the cotton rat are of considerable interest to the field of nutrition as related to dental caries. Primarily they seem to point to the importance of the cotton rat as an experimental animal for the study of dental caries. Whether such experimental studies can help directly to elucidate the relation of the numerous factors involved in the etiology of dental caries in man is unknown. However if systematic, carefully controlled studies can be made in experimental animals to determine the role of various factors related to the etiology and control of the experimental forms of dental caries, a more fruitful research program could be planned to answer the many controversial issues about dental caries in man. An investigation of the relation of increasing the fat and protein fractions of the ration at the expense of the carbohydrate upon the caries incidence in the cotton rat would be a valuable contribution. In this way it might be possible to produce a simplified ration which is more comparable to human diets. Thus a better starting point for the study of methods of controlling experimental dental caries would be established.

HUMAN BIOASSAY TECHNIC AND APPLICATIONS

Much of the foundation of human nutrition rests on animal experimentation, and on the assay of food provided by chemical and bacteriologic methods. Criticism is occasionally expressed that animal assays are not always directly applicable to problems of human nutrition, that preparation of a food sample for microbiologic or animal assay may not be duplicated during digestion in man, and that the specific depletion induced in animals prior to assay may be quite different from the complex condition underlying nutrition in man. A technic to assay or to determine the availability of

vitamins directly on man would obviate some of this criticism and be of direct value to human nutrition.

D. Melnick, M. Hochberg, and B. L. Oser (*J. Nutrition* **30,** *67* (*1945*)) have studied the conditions for a satisfactory human bioassay which is based on the fact that in normal human subjects the urinary excretion of water soluble vitamins is proportional to the quantity of vitamin consumed. A precision for the human bioassay comparable to animal assay is claimed, and since the test material is fed in a single dose, perishable foods may be assayed without question as to the stability of the product over a prolonged test period.

In their first paper these investigators have outlined the technic and its experimental justification as applied to ascorbic acid, thiamine, riboflavin, and nicotinamide. Total ascorbic acid, free thiamine, riboflavin, and N′-methylnicotinamide are determined in a suitably preserved twenty-four hour specimen of urine. Normal subjects on a basal diet supplying considerably more than the minimum vitamin needs are employed as test subjects. Selection of the subjects is based on a previous history of adequate diet and a direct testing for normal vitamin clearances after test doses. Five such subjects are generally employed for each assay, and are placed on the special basal diet only during the two day period required for testing the urinary response during a control period and to a standard amount of vitamin.

In an example of the technic given for ascorbic acid, a basal twenty-four hour urine was collected for analysis, and a second period collection after the administration of a 200 mg. dose of ascorbic acid in solution form. An average of 48.6 per cent of the test dose was recovered in the 5 subjects. Two weeks later the procedure was repeated with the ascorbic acid being replaced by vitamin C tablets. In this series an average of 48.0 per cent of the test dose was excreted indicating nearly 100 per cent utilization of the ascorbic acid in these tablets. There was considerable individual variation in excretion levels, particularly in the basal levels of excretion, but these did not seem to lead to great error in the final assay results. The variations with ascorbic acid were claimed to be greater than with the other vitamins. Although considerable differences in basal excretion values for the same individual may be noted over extended intervals, satisfactory evidence of the constancy of this excretion over a short period is presented to justify its use in the calculation of the percentage of "test dose excreted." Graphic illustration is also provided to show that a linear response of extra vitamin excreted in the urine is obtained with increasing test dose of vitamin. In general these investigators conclude that the reproducibility of data obtained in the human bioassay is superior to that encountered in most animal assays.

This general technic has been employed previously to determine the availability of B-vitamins to man. D. Melnick, W. D. Robinson, and H. Field (*J. Biol. Chem.* **138,** *49* (*1941*)) showed that the increased thiamine requirement of peptic ulcer patients was due to the antacid medication, H. T. Parsons and J. Collord (*J. Am. Dietet. Assn.* **18,** *805* (*1942*)) that the B-vitamins in fresh yeast were only partially available, and A. Williamson and H. T. Parsons (*J. Nutrition* **29,** *51* (*1945*)) that crude fiber in the ration did not reduce the availability of thiamine. D. Melnick, M. Hochberg, and B. L. Oser (*Ibid.* **30,** *81* (*1945*)) have now studied the interesting question of whether the thiaminase (thiamine destroying enzyme) of raw fish consumed in the human dietary might destroy significant amounts of thiamine in the diet. The action of this enzyme in the raw fish of fox rations, it may be recalled (*Nutrition Reviews* **1,** *61*, *403* (*1943*); **2,** *128* (*1944*)), may lead to acute thiamine deficiency (Chastek paralysis).

In vitro experiments with several raw fish products revealed the presence of the thiamine destroying factor in raw clams, and this food was chosen for a study of its effect on thiamine availability in man. Preliminary *in vitro* experiments showed that both the natural and added thiamine in a homogenized basal diet were readily destroyed by admixture with ground raw clams, in contrast to a cooked clam preparation which permitted relative stability of thiamine.

In evaluating the effect of ingesting raw clams on the availability of thiamine, the general technic of the human bioassay was applied. After establishing basal excretions of thiamine and the percentage of a test dose of 7.5 mg. excreted, the subjects consumed the average serving of 100 g. of raw clams, divided between three consecutive meals. There was a distinct difference in the excretion of thiamine depending on whether or not the clams were cooked. Assay figures indicated that 42 per cent of the test dose of thiamine and about 50 per cent of that in the basal diet was destroyed as a result of ingestion of the raw clams. The investigators feel that these results warrant "consideration of the anti-thiamine principle in fish products as a possible conditioning factor in malnutrition." The seriousness of this statement of course depends on the frequency with which raw clams are consumed, but the facts are illustrative of food incompatabilities which would not be revealed by chemical analysis of individual foods.

Another possible case in which destruction of a vitamin may occur due to an enzyme agency of a food is that of the commonly occurring ascorbic acid oxidase of vegetables. Using a procedure similar to that previously described, the effect of dietary ascorbic acid oxidase has been studied by M. Hochberg, D. Melnick, and B. L. Oser (*J. Nutrition* **30,** *193 (1945)*). *In vitro* studies of homogenized diets showed that extensive destruction of ascorbic acid may occur, and that if incubation were sufficiently prolonged, much of the initially formed dehydroascorbic acid was converted into biologically inactive products. In the *in vivo* studies, the enzyme was supplied by a liberal quantity of raw green vegetables. In control experiments the vegetables were cooked. The results fail however to show any effects of the raw vegetables on the availability of the dietary or added ascorbic acid under the usual eating conditions. Apparently either the enzyme is quickly inactivated in the stomach upon disintegration of the vegetable, or if enzyme action occurs, destruction of the vitamin beyond the biologically active dehydro stage is insignificant.

In a third application of their technic, M. Hochberg, D. Melnick, and B. L. Oser have studied the availability for human nutrition of the thiamine in live yeast (*J. Nutrition* **30,** *201 (1945)*). Their results confirm in general the earlier work of Parsons and Collord (*loc. cit.*) that only a small fraction of such thiamine is directly available. In the particular yeast preparation used, which had a high total thiamine content mostly in the free form (nonphosphorylated), only 17 per cent was available. Extended periods of urine collection and tests with divided doses of pure thiamine offered no support for the possibility that the low recovery was due to a slower liberation of thiamine from the yeast and more efficient utilization.

There is no doubt that the human bioassay provides a direct answer to many problems of human nutrition not satisfactorily solved by chemical analysis or animal assay.

THE CECAL FLORA AND NICOTINIC ACID SYNTHESIS

The phenomenon of refection in the rat has long been associated with the distention of the cecum by a mass of actively fermenting material, in which it is believed that the

vitamins missing from the diet are synthesized by the microorganisms present and then absorbed by the host. The work of P. Ellinger, R. A. Coulson, and R. Benesch (*Nature* **154,** *270 (1944)*) and of P. Ellinger, R. Benesch, and W. W. Kay (*Lancet* **I,** *432 (1945)*) suggested that in man the flora of the intestinal tract may be able to synthesize nicotinic acid which can subsequently be absorbed from the gut.

The evidence supporting this view was circumstantial but strong. Human subjects on a steady diet excreted less N′-methylnicotinamide (the principal end product of nicotinic acid metabolism) after receiving doses of sulfonamides to reduce the intestinal flora than in the period before dosing. On cessation of the administration of sulfonamide the excretion of N′-methylnicotinamide rose again.

R. Benesch (*Lancet* **I,** *718 (1945)*) has since used the opportunity which was offered by a patient with a cecostomy to study this problem more directly. The mixed cecal flora was sampled by washing out the cecum with water, shaking to form a uniform suspension. A series of flasks containing 150 ml. of a Bacto-Peptone medium were each inoculated with 1 ml. of the bacterial suspension and then incubated, one half of them aerobically and the other half anaerobically. At intervals up to nine days after inoculation 2 flasks from each set were removed from the incubator and the contents of the flask analyzed for nicotinic acid.

Analysis of the contents immediately after inoculation showed that the medium contained 0.4 microgram nicotinic acid per milliliter. In the aerobic culture the nicotinic acid content rose rapidly until the sixth day, when it was between five and six times as great as at the time of inoculation. In the anaerobic culture on the other hand the nicotinic acid content fell to about one half of the initial value. These figures refer to the total nicotinic acid, both that in free solution in the medium and that contained within the cells of the microorganisms present.

After nine days the number of organisms in each type of culture was measured and found to be of the same order. There were about five times as many organisms per milliliter in the anaerobic as in the aerobic cultures. The species of bacteria present in the original sample from the cecum were also determined and listed.

The results with the aerobic cultures show definitely that this human cecum did contain organisms capable of synthesizing nicotinic acid, thus supporting the earlier conclusion of Ellinger and co-workers that "biosynthesis" of this vitamin can occur in the human intestine. An unexpected feature of the experiment was the finding that nicotinic acid was destroyed in anaerobic culture. It is of course known that some bacteria can destroy nicotinic acid, for example certain species of soil bacteria observed by M. J. C. Allinson (*J. Biol. Chem.* **147,** *785 (1943)*), but the observation of destruction by cecal organisms is new.

These findings afford a basis for speculation by Benesch on the utilization of nicotinic acid by the human host and its absorption from the gut. He suggests that there is in the bowel an ecologic system in which aerobic and anaerobic organisms live together in a shifting equilibrium, which will determine the net amount of nicotinic acid made available to the host by the intestinal flora. It is therefore possible that in a good many cases where deficiency disease is thought to have begun with an interference with absorption of the water soluble vitamins, due to some abnormal condition of the intestinal mucosa, a disturbance in the equilibrium between the various species of bacteria normally present may have occurred, leading to a decrease in the amount of nicotinic acid made available to the host. Thus the maintenance of a "normal" intestinal flora may play an important part in protection from deficiency diseases. This

may be particularly true of nicotinic acid deficiency, since it is known that some populations among whom pellagra is rife ingest in their food considerably more nicotinic acid than other populations among whom the disease is rare.

ACTION OF INSULIN

Vitamins and hormones are intimately related in that both groups of substances exert profound physiologic effects in trace amounts. In the case of the vitamins, thiamine, riboflavin, niacin, and more recently pyridoxine (*Nutrition Reviews* **3,** *72, 343 (1945)*), a specific biochemical function has been discovered in their role as coenzymes, while in the case of hormones the first definitive biochemical role has only now been announced from Cori's laboratory (W. H. Price, C. F. Cori, and S. P. Colowick, *J. Biol. Chem.* **160,** *633 (1945)*). These workers have demonstrated that the action of the enzyme hexokinase, which with adenosine triphosphate converts glucose to glucose-6-phosphate, is inhibited either *in vivo* or *in vitro* by anterior pituitary extract, and that this inhibition can be counteracted by insulin.

Such a discovery, apart from its general importance in the hormone field, its implication to medicine, and the research stimulus it naturally incites, is of special interest to biochemists and physiologists in its relation to the background of research which preceded it. The intensive efforts of many investigators to elucidate the enzymatic reactions involved in the combustion of carbohydrate is widely recognized. As by-products of these efforts have come the isolation of seven crystalline enzymes and an increased understanding of the role of thiamine, niacin, and riboflavin in carbohydrate metabolism. A central theme established in this work is that the formation of phosphate derivatives is necessary before the metabolism of glucose can proceed. Cori and associates have been outstanding in this particular phase of work, and it is fitting that the discovery of a fundamental biochemical defect in diabetes and the role of insulin should come to this laboratory. The recognized positive action of insulin in promoting glycogen synthesis and its effect on carbohydrate metabolism have indicated a specific "accelerator" role. The work of E. Bueding, J. F. Fazekas, H. Herrlich, and H. E. Himwich (*Science* **95,** *282 (1942); J. Biol. Chem.* **148,** *97 (1943)*) and of D. Klein (*Ibid.* **145,** *35 (1942)*) appeared to isolate the biochemical site of insulin action to the chemical steps from glucose to pyruvate formation. No doubt each investigator, including Cori, working with enzyme systems involved in carbohydrate metabolism, has tested for an insulin effect. In general these tests have led either to a negative outcome or to disputed and questionable results. In retrospect these simple experiments failed because of lack of consideration of physiologic and clinical work which indicated a complex relation of endocrine functions, specifically an antagonism of the anterior pituitary and the pancreas.

Opie called attention to the prevalence of hyaline degeneration of the islands of Langerhans of the pancreas in diabetes, and this has been considered as the classic pathologic lesion of this disease. It became slowly evident however that this lesion often occurs in later life among nondiabetics, that the extent of the lesion need not bear any close relation to the severity of the disease, and finally that in some diabetics the pancreas appears perfectly normal. Theories of diabetes arose which stressed not the lack of insulin but an "insensitivity to insulin," and with the classic work of Houssay on the diabetogenic principle of the pituitary, led to the general concept

that a relative excess of pituitary hormone rather than absolute lack of insulin may be the more fundamental hormonal disturbance in diabetes. In contrast to the action of insulin, anterior pituitary extracts result in hyperglycemia and glycosuria. The antagonistic action of insulin and the anterior pituitary hormone was vividly demonstrated in the "Houssay dog," lacking both the pancreas and anterior pituitary, in which fairly normal carbohydrate balance was attained (although such animals are extremely sensitive to the action of either hormone).

In retrospect, therefore, the crucial experiments of Price, Cori, and Colowick seem to be in logical sequence. In their preliminary announcement, these investigators report that the formation of glucose-6-phosphate from glucose (hexokinase reaction) can be inhibited by anterior pituitary extract, either by adding the hormone to the enzyme preparation *in vitro* or by injecting rats with the pituitary extract and subsequently measuring the activity of the extracted tissue hexokinase. Insulin counteracted this inhibition, again whether injected into the animal or when tested *in vitro*. Employing phosphate-saline extracts of rat muscle, liver, kidney, heart, and brain tissue as the hexokinase preparation, it was noted that the inhibition by anterior pituitary extract was released with time. With purified hexokinase preparations the inhibition was prolonged. Although insulin releases hexokinase from the inhibition by anterior pituitary extract, insulin does not itself enhance hexokinase activity under the experimental conditions employed.

It seems certain that the *in vivo* effect of insulin is similar to the *in vitro* action since (1) muscle extracts prepared from diabetic rats after the injection of insulin show normal hexokinase activity, and (2) rats made diabetic by the injection of alloxan yielded tissue extracts which showed the same hexokinase activity as those obtained from rats previously injected with anterior pituitary extract. The action of anterior pituitary extract and insulin on the enzyme hexokinase provides for the biochemist and physiologist a most fascinating and mechanistic picture of the clinical picture of the diabetic complex.

The chemical interrelations of hexokinase, anterior pituitary extract, and insulin become of special interest as a result of these experiments. It has been known that insulin is inactivated when reduced by cysteine, and Price, Cori, and Colowick report that such a treated insulin also loses its antagonistic effect toward inhibition of hexokinase by anterior pituitary extract. Since insulin is itself a disulfide protein (S-S), and the activities of several enzymes are known to be dependent on their SH or S-S groups, it seems possible that both hormones exert their activity by affecting the sulfhydryl balance of hexokinase, although a direct action of insulin on the anterior pituitary hormone is not excluded.

THIOURACIL AND LIVER CIRRHOSIS

The relation of methionine and other sulfur amino acids to liver necrosis, both the toxic type (toxipathic) and the dietary type (trophopathic), has been previously reviewed (*Nutrition Reviews* **2,** *340 (1944)*). In rats methionine delays the production of dietary cirrhosis and cystine accelerates the condition.

P. György and H. Goldblatt (*Science* **102,** *451 (1945)*) have reported that the addition of thiouracil to a cirrhosis-producing synthetic diet has a preventive effect on the development of cirrhosis in rats. This study was stimulated by the general relation of the sulfur-containing amino acids to cirrhosis, and by a previous finding that

thiourea, in spite of severe toxic action, appeared to possess a prophylactic effect on liver cirrhosis. Thiouracil possessed particular interest in view of its action of decreasing thyroid function, and the known association of liver lesions with hyperthyroid conditions. The investigators postulate that thiouracil might have a beneficial effect on the liver, as a result of a general decrease in metabolic rate, including that of the protective methionine.

Two groups of 14 adult male rats were fed a diet containing casein, Crisco, sucrose, salt mixture, vitamins, and a daily supplement of 50 mg. of cystine to accelerate the liver cirrhotic changes. One group received in addition 0.1 per cent thiouracil in the diet mixture. The experimental period was one hundred fifty days, with animals surviving this period sacrificed for examination of the liver. Weight losses were less in the group of rats which received thiouracil, and 12 of the 14 rats not receiving the drug were dead before the end of one hundred fifty days as compared with the survival of 12 of 14 of the thiouracil treated rats. Microscopic examination of different lobes of the liver revealed the absence of cirrhosis in 9 of the thiouracil treated rats, and in but one of the control rats. Ten of the control rats showed a severe cirrhosis as contrasted to only one of the thiouracil treated rats showing this degree of liver damage. The typical hypertrophy and hyperplasia of the thyroid caused by thiouracil was noted in the treated rats. Daily food intakes of the rats were recorded, and these appear sufficiently alike to eliminate food intake as a possible factor influencing the results.

The investigators refer to the protective action of sulfanilamide against the toxipathic cirrhosis caused by carbon tetrachloride (J. C. Forbes, B. E. Leach, and G. Z. Williams, *Proc. Soc. Exp. Biol. Med.* **51,** *47 (1942)*), and the action shared by both sulfanilamide and thiouracil in inhibiting thyroxin formation (C. G. MacKenzie and J. B. MacKenzie, *Endocrinology* **32,** *185 (1943)*; E. B. Astwood, J. Sullivan, A. Bissell, and R. Tyslowitz, *Ibid.* **32,** *210 (1943)*). It is suggested therefore that the protective action of these compounds on the liver is indirectly through their thyroid action in lowering the metabolic rate of methionine. Since methionine protects against both dietary and toxipathic cirrhosis, it seems possible that thiouracil would be beneficial in both cases. A direct testing of thiouracil for protection against toxipathic cirrhosis has not yet been made.

The use of thiouracil as a supportive measure along with a high protein and methionine rich diet may therefore be investigated in the treatment of cirrhosis.

TOPICALLY APPLIED FLUORIDE AND DENTAL CARIES

One of the most hopeful means of decreasing the high incidence of dental caries is through the use of fluorides. It has been repeatedly observed that persons residing in areas supplied with water which naturally contains fluorides show a lower incidence of caries than those from fluoride free areas (*Nutrition Reviews* **2,** *149 (1944)*). It seems to be well established that teeth which develop under conditions which lead to the deposition of a certain amount of fluoride in the structure of the enamel are more resistant to caries than would otherwise be the case. It would be even more useful if such protection could be brought about through the application of the fluoride to the surface of the tooth after enamel formation is complete. J. W. Knutson and W. D. Armstrong have been studying the caries preventive effects of topically applied fluoride in a group of Minnesota schoolchildren, and have previously reported (*Pub. Health Reports* **58,** *1701 (1943)*) encouraging results from the first year's observations. A report

of the findings for the second study year has now been published (*Ibid.* **60,** *1085 (1945)*). They confirm and extend the previous conclusions.

A group of 289 children, 7 to 15 years of age, received 8 to 15 four-minute treatments with 2 per cent sodium fluoride during a period of eight weeks at the beginning of the study. The surfaces of all teeth in the upper and lower left quadrants of the mouth were treated while those in the right quadrants were left untreated for controls. In addition, all the teeth of about 400 children in the same school served as additional controls. Each child in the two groups received a dental examination and caries evaluation at the beginning of the study and at yearly intervals. The second annual period has now elapsed.

Over the entire two year period the caries incidence was 41.3 per cent less for the fluoride treated teeth than for the untreated teeth of the experimental group of children. For the second year only, the incidence was 46.6 per cent less, thus indicating that the treatments were fully as effective in the inhibition of dental caries during the second year following treatment as during the first year. Although it appears that noncarious surfaces of teeth which were already carious (in other areas) at the time of treatment were also protected, the degree of protection was only 20 per cent, or about half of that observed for the teeth which were caries free up to the time of treatment. There was no evidence that the treatment was effective in the control of active caries but rather that the effect is mainly one of prevention. The incidence of caries in the control group, by mouth quadrants, was strikingly similar to the corresponding untreated quadrants in the treated group.

The authors' conclusion that this "pilot study on the caries inhibitory effect of topically applied fluorides is sufficiently encouraging to justify additional and more stringently controlled studies" seems well justified. An important point in connection with the practical and wide use of such a method for the prevention of dental caries concerns the minimum number of applications necessary for effectiveness. Studies of this type are now in progress.

NUTRITIONAL ASPECTS OF JAUNDICE IN NEGROES WITH PNEUMONIA

E. L. Turner *et al.* (*Proc. Central Soc. Clin. Res.* **15,** *27 (1942); South. Med. J.* **36,** *603 (1943)*) reported an unusually high incidence of jaundice in Negroes with pneumonia. Their patients were seen in the years 1936 to 1938 when there was much unemployment among Negroes in Nashville, and it was assumed that nutrition had suffered as a consequence, though no clinical or dietary studies were undertaken to establish this point. Since the occurrence of icterus in 67.7 per cent of their 74 cases appeared far out of line with any possible racial predisposition to jaundice in pneumonia, the idea of latent hepatic damage from a high fat diet relatively poor in protein and vitamin B-complex factors was attractive. Turner and associates therefore gave a deficient diet to a number of dogs and then induced pneumonia in these animals and in controls. Although the mortality was similar in the two groups, all dogs on the deficient diet developed positive Van den Bergh reactions within four days of the induction of pneumonia. These findings have been discussed previously (*Nutrition Reviews* **2,** *42 (1944)*).

As an extension of these observations M. J. Bent, E. L. Turner, G. D. Holloway, and J. R. Cuff (*South. Med. J.* **38,** *730 (1945)*) compared the effects of induced pneumonia in two groups of dogs on a diet of fat "side meat," boiled turnip greens, corn meal, and sorghum, the control group having

a supplement of brewers' yeast. Following inoculation all of the 9 dogs on the unsupplemented diet developed pneumonia and a positive Van den Berg reaction. Two of the 11 dogs on the supplemented diet failed to develop pneumonia and in no instance did the Van den Bergh reaction become positive. The meaning of these results is uncertain since there was no effort at paired feeding and the dogs not receiving yeast lost weight whereas the others tended to gain weight during the three to seven weeks on the diet before pneumonia was induced. Thus inanition or any of several specific factors in the vitamin B-complex may have been responsible for the significant differences observed. Marked fatty degeneration of the liver was found in the dogs on the unsupplemented diet. Further extension of this work with paired feeding and supplementation of the diet with various pure compounds should clarify the mechanism of jaundice in undernourished dogs with induced pneumonia.

It is difficult to be sure of the exact application of these observations to the problem of jaundice in clinical pneumonia. In the absence of precise dietary history which at best is likely to present only fragmentary information, and wanting chemical determination of vitamin levels and contents, it cannot be stated that an ill chosen diet was responsible for the jaundice in the patients with pneumonia. One would certainly have to eliminate acute or chronic alcoholism and perhaps other factors. Since studies in Birmingham and Augusta (W. B. Bean, T. D. Spies, and M. A. Blankenhorn, *Medicine* **23,** *1 (1944);* V. P. Sydenstricker and E. S. Armstrong, *Arch. Int. Med.* **59,** *883 (1937)*) have shown that pellagra is relatively uncommon in Negroes in those areas, further data are required before a malnutritional cause for jaundice is established for the Nashville Negroes with pneumonia. The animal experiments make the problem attractive for further study in man.

GROWTH FACTORS FOR BLOOD AND TISSUE FLAGELLATES

The contributions of bacteriology to the science of nutrition have been extensive and promise to increase. Microbiologic methods have been perfected to the point where they can give the same kind of information that the longer, more complicated, and more costly experiments on animals have produced in the past. A little explored field, however, is that of the specific nutrients required for organisms slightly more advanced in the evolutionary scale than bacteria and molds (*Nutrition Reviews* **2,** *133 (1944)*). Therefore, the report of H. A. Senekjie and R. A. Lewis (*Am. J. Trop. Med.* **25,** *345 (1945)*) upon the growth factors of certain blood and tissue flagellates is of note. The organisms studied were various leishmanias and Trypanosoma cruzi. It was first established that when leishmania broth was used, thiamine, niacin, and pyridoxine had temporary growth stimulating properties, ascorbic acid had no effect, while riboflavin and calcium pantothenate retarded growth in the organisms. A number of different blood constituents were then used to determine which contained the stimulating factors known to be present in whole blood. The mediums employed included leishmania agar rabbit serum medium, leishmania agar washed rabbit erythrocyte medium, leishmania agar egg white medium, leishmania agar hemoglobin medium, leishmania blood phenol agar, leishmania albumin agar, leishmania euglobulin, and leishmania pseudoglobulin agar. In addition mediums were prepared using various combinations of globulin, euglobulin, and pseudoglobulin. L. donovani, L. brasiliensis, L. tropica, and T. cruzi were tested on these mediums. Details of the numerous procedures and

variations should be consulted in the original paper.

It was found that the growth promoting factors essential for the flagellates were absent in hemoglobin and in erythrocytes washed free of serum, but were present in serum. The factor or factors were partially thermostable when heated to 70°C. for thirty minutes but were destroyed at 100°C. They were not present in the albumin, euglobulin, or pseudoglobulin fractions of rabbit serum, singly or in combination. Addition of thiamine, niacin, para-aminobenzoic acid, and pyridoxine to hemoglobin did not produce the growth promoting effect of serum. Since the essential stimulant was dialyzable it may have been lost during the process of fractionating the serum.

The main conclusion these authors reach for this investigation is that hemoglobin is not necessary for the growth of these organisms and therefore the descriptive name "hemo flagellates" is inappropriate. M. Lwoff (*Compt. rend. Soc. de biol.* **130,** *406 (1939)*) had reported that ascorbic acid, hematin, and an unknown fraction of blood serum were essential for growth and multiplication of flagellates. It is not possible from the data presented to explain the discrepancy in these two sets of observations and final judgment must await further investigation which has as its aim the precise identification of the factor or factors which stimulate growth of flagellates. Use of the various fractions of human plasma obtained by E. J. Cohn and his colleagues (*Nutrition Reviews* **3,** *103 (1945)*) might give further clues, and a study of amino acids should be of use.

FAT AND CHOLINE IN THE PRODUCTION OF HEMOLYTIC ANEMIA

The ingestion of excessive quantities of fat results in the destruction of red blood cells in dogs (A. Loewy, L. W. Freeman, A. Marchello, and V. Johnson, *Am. J. Physiol.* **138,** *230 (1942)*). Dogs with bile fistulas receiving alternately high and low fat diets over an extensive period of time excreted increased amounts of bile pigments during the period on high fat diets, indicating an increased destruction of red blood cells. However, no significant anemia was detected in any animal. Weekly blood cell counts fluctuated and did not correlate with the bilirubin output.

J. E. Davis (*Am. J. Physiol.* **142,** *213 (1944)*) has also reported that 3 dogs receiving 60 g. of lard daily exhibited no significant changes in erythrocyte counts. He showed, however, that with the simultaneous feeding of high fat and choline chloride to dogs a sudden large destruction of erythrocytes results. Concurrently, J. E. Davis (*Ibid.* **142,** *402 (1944)*) has demonstrated the production of hyperchromic anemia in dogs by long term feeding of progressively increased doses of choline chloride without elevated fat intakes.

The production of hemolytic anemia in both dogs and human beings by feeding simultaneously two normally required dietary factors, fat and choline, is reported by J. E. Davis and J. B. Gross (*Am. J. Physiol.* **144,** *444 (1945)*). Three normal dogs were given 60 g. of "Vegetole" (a mixture of vegetable and animal fats) and 10 mg. of choline chloride per kilogram of body weight twice daily for four consecutive days. The data show an initial large drop in red blood cell counts of 20 to 37 per cent which is accompanied by smaller decreases in hemoglobin values. Continuation of feeding for four days showed little further decrease in red blood cells. The icteric index increase of two or three times the initial value during the first two test days indicates red blood cell destruction. Values for the later test days are lacking. Failure of the reticulocytes to increase during the period

when "erythrocyte numbers showed a tendency to rise" after fat feeding was discontinued seems paradoxical. However, 2 human subjects who received one quarter of a pound of butter and 400 mg. of choline chloride with breakfast showed an increase in reticulocytes three to five days following the experimental meal. The fluctuations in red cell counts on these subjects make any interpretations difficult. It is difficult to understand the rapid return to normal counts.

Comparison of the doses of choline chloride administered in these experiments with those required for the prevention of fatty livers in animals is of interest. C. Entenman and I. L. Chaikoff (*J. Biol. Chem.* **138,** *477 (1941)*) have shown that to prevent fatty livers in dogs, 35 mg. or more per kilogram of body weight are required daily. This is approximately twice the amount used by Davis and Gross. It has been found (H. J. Channon, J. V. Loach, and G. R. Tristram, *Biochem. J.* **32,** *1332 (1938)*) that rats need 10 to 20 mg. of choline per day. Based on 10 mg. for a rat weighing 100 g., a human being weighing 60 kg. would require 6 g. daily, which is fifteen times the dosage given to the 2 human subjects. It is difficult to reconcile the marked effects obtained by Davis *et al.* with the small doses of choline administered with fat. However, it would be important to establish with comprehensive data whether the dietary essential, choline, when fed in various quantities with excessive fat would consistently produce such an effect.

PANTOTHENIC ACID, THE ADRENAL CORTEX, AND GRAY HAIR

The underlying causes of achromotrichia (graying of the hair) are not well understood. Both dietary deficiencies and hormonal disturbances have been implicated. A deficiency of pantothenic acid is known to result in graying of the hair in rats and other experimental animals and there is evidence that additional factors including para-aminobenzoic acid may at times be involved. On the other hand there is no reason to believe that human achromotrichia is caused by a dietary deficiency (*Nutrition Reviews* **2,** *90 (1944)*). In man, the processes of graying and of aging are frequently associated and there is a popular belief that graying may be accelerated by emotional stress. It is not surprising, therefore, that hormonal control of the graying process should have been surmised even though direct experimental evidence has in large measure been lacking. This confused picture has been somewhat clarified by the investigations of Ralli and associates who have shown that even in experimental animals rendered gray by a deficiency of pantothenic acid, adrenalectomy will bring about the restoration of the original color of the hair.

E. P. Ralli and I. Graef (*Endocrinology* **32,** *1 (1943)*) investigated the effect of adrenalectomy on the color and growth of hair of rats at various stages of pantothenic acid deficiency. Animals on identical diets but not adrenalectomized served as controls. After eighteen to one hundred eight days on experiment the backs of all rats were shaved and a number of animals on each diet were adrenalectomized. By periodic observation of the shaved areas, the effects of adrenalectomy on four general classes of animals were observed; those with an early deficiency of pantothenic acid, those with a well developed deficiency, those recovering from such a state, and those given adequate amounts of the vitamin throughout the experiment.

When the animals were shaved it was noted that the skin of those ingesting the deficient diet was uniformly pink while the backs of the animals receiving adequate amounts of pantothenic acid bore pigmented

bands. The effect of adrenalectomy was (a) to accelerate the growth of new hair in all animals, (b) to restore the original color of the hair and to bring about repigmentation of the skin in animals deprived of pantothenic acid, and (c) to accelerate the recovery process in animals given pantothenic acid after a period of deprivation. In addition there developed a generalized bluish coloration of the skin after adrenalectomy which was not apparent in animals with intact adrenals. This diffuse pigmentation, due to widespread accumulation of melanin in hair bulbs and follicles, reached a maximum intensity at about the sixteenth day after the operation and then began to fade. One to two months after adrenalectomy the color of the skin was normal. The observation concerning the effect of adrenalectomy on rate of hair growth confirmed the earlier observation of E. O. Butcher and R. A. Richards for albino rats (*Endocrinology* **25,** *787* (*1939*)).

Ralli and Graef found from histologic studies that the graying of the hair was associated with atrophy of the hair apparatus and a cessation of melanin deposition. Following adrenalectomy there was a marked transitory hyperplasia of the hair bulbs and follicles and an increased deposition of melanin. These changes were apparent in all adrenalectomized rats, whether given a deficient or a complete diet, but they appeared to be of shorter duration in the deficient animals.

E. P. Ralli and I. Graef (*Endocrinology* **37,** *252* (*1945*)) also studied the antagonistic action of adrenal hormones in similar adrenalectomized rats. In these experiments all animals were given the pantothenic acid deficient diet and after twenty-eight to thirty-two days all were adrenalectomized. Some animals were then continued on the deficient diet while others were given in addition a rice bran extract or pantothenic acid. During the period following adrenalectomy some animals from each dietary group were given daily injections of desoxycorticosterone acetate or an extract of the naturally occurring cortical hormone.

Desoxycorticosterone acetate in large measure prevented the effects of adrenalectomy on the color and growth of the hair. In animals maintained on the deficient diet, the injections of this hormone prevented melanin deposition and prevented the regrowth of fur over the area shaved at operation. The effects of injections of extracts of adrenal cortex though similar were less striking, probably because a somewhat smaller dosage was used.

Other observations of Ralli and associates suggest that dehydration and the concentration of sodium chloride in body fluids influence melanin production or deposition but the relationships are not entirely clear. Ralli and Graef observed that 2 per cent sodium chloride in the drinking water of normal rats on complete diets improved the pigmentation of the fur and increased melanin deposition in the hair follicles, as evidenced by diffuse bluish coloration of the skin. H. J. Spoor and E. P. Ralli (*Endocrinology* **35,** *325* (*1944*)) found, however, that dehydration either by water deprivation or by 2 per cent sodium chloride produced chemical changes in the skin simulating those induced by pantothenic acid deficiency.

These interesting researches throw much new light on the physiologic processes involved in graying of the hair. It appears that hormone balance, dietary deficiencies, and possibly mineral and water balances may influence the production of melanin or its deposition in the hair and skin. The hormonal control must be particularly strong since adrenalectomy overcomes the effect of a pantothenic acid deficient diet. It is not as yet clear, however, whether the various factors operate through single or separate mechanisms and the nature of this process or processes is not well characterized.

USE OF URINARY VITAMIN EXCRETION VALUES IN NUTRITIONAL SURVEYS

Measurements of the urinary excretion of the water soluble vitamins have aided greatly in evaluating the nutritional status of population groups. Absolute figures of excretion levels to divide sufficiency and deficiency states are still lacking because of individual variation and the difficulty in defining the "subclinical" deficiency state, but in general the urinary excretion of a water soluble vitamin appears to reflect its body stores. There have been various claims for the advantages of different methods of urine sampling, such as the use of fasting specimens, random specimens, or collection of a sample after administration of a test dose of vitamin (oral loading test). Some investigators look with disfavor upon urine collection because of technical uncertainties and possible complication by kidney function differences, and therefore advocate blood analysis. It is evident that technic preferences are dependent on the experimental situation, and that the control afforded by a bed patient in the hospital may be very different from the conditions prevailing in a field survey. Apart from these considerations there has been a lack of data to determine which of the tests actually reflect most accurately a change in dietary status. Toward correcting this situation the report of R. E. Johnson, C. Henderson, P. F. Robinson, and F. C. Consolazio (*J. Nutrition* **30,** *89 (1945)*) is of value.

These investigators studied the comparative merits of vitamin analyses of fasting, random, and "oral loading" specimens of urine in groups of soldiers under the relatively well controlled conditions afforded by military camp life. Attention was directed to the ascorbic acid, thiamine, riboflavin, and N′-methylnicotinamide content of urine. In the first series of experiments the effects of repeated load tests were studied. Fifty soldiers on a combat ration providing estimated daily averages of 65 mg. of ascorbic acid, 1.5 mg. of riboflavin, 2.0 mg. of thiamine, and 17 mg. of niacin were given the load test initially, and the same test three and five weeks later. A second group of 46 soldiers was given the test at the five week mark. On the morning of the test day, and after collecting a fasting specimen of urine, each subject drank 150 ml. of a solution containing 500 mg. of ascorbic acid, 5 mg. of riboflavin, 5 mg. of thiamine hydrochloride, and 50 mg. of nicotinamide. Four hours after this solution was administered, urine was again collected for analysis. Breakfast was taken during the four hour period.

Analysis of the fasting and four-hour urines for ascorbic acid, thiamine, riboflavin, and N′-methylnicotinamide showed that the average excretion of each substance was essentially the same in the two groups. Although the range of excretions for the group which had received two previous load tests tended to be somewhat greater, the distribution curves were not greatly different. The authors concluded that neither the fasting urine values nor the results of loading tests were affected by previous loading tests. This conclusion is of course valid only for the dietary conditions that prevailed; in deficient subjects the previous loading tests might have substantially affected the body stores of the vitamin and hence the urinary excretion on subsequent tests.

The distribution curves for the four-hour excretion levels of each vitamin are informative with regard to the difficulty of establishing a narrow range of excretion values which might define dietary sufficiency in a given individual. Thus, 30 per cent of the group showed an excretion of 40 to 60 mg. of ascorbic acid, 10 per cent excreted as little as 1 to 20 mg., while 20 per cent excreted more than 80 mg.

In a second series of experiments a comparison was made of fasting urinary levels

with the results of so-called load tests. In these experiments two groups of 26 and 31 men were employed, all on the adequate field ration providing 140 g. of protein, 90 mg. of ascorbic acid, 2.5 mg. of riboflavin, 2.1 mg. of thiamine, and 27 mg. of niacin. After initial determinations of the fasting and "load test" excretions of ascorbic acid, thiamine, riboflavin, and N′-methylnicotinamide, one group received vitamin pills and the other placebos for a three week period. The vitamin pills provided an extra daily amount of 112.5 mg. of ascorbic acid, 4.5 mg. of riboflavin, 30 mg. of nicotinamide, 3 mg. of thiamine hydrochloride, 7500 U.S.P. units of vitamin A, and 600 U.S.P. units of vitamin D. After the three week experimental period, fasting and "load test" excretions were again determined in the two groups. Examination of the data showed clearly that the fasting specimen was a much more sensitive index for determining whether the individuals had received extra vitamins than was the "load test" excretion. The group which had received placebos yielded satisfactory check values for fasting urinary excretions, while the average fasting urinary excretions of those receiving the extra vitamins were approximately doubled. The "load test" urinary values showed neither satisfactory checks with the group receiving placebos, nor distinct rises of excretion in the group which received vitamin pills. The results clearly favor the use of fasting urinary excretion values over the "load test" technic.

In a third series of experiments, samples of blood (for hemoglobin, serum protein, chloride, and ascorbic acid) and urine were collected in a series of 12 subjects at various times of the day to determine whether a random collection of samples might replace the more careful control required for fasting specimens and load testing. Postprandial samples of blood and urine were collected in such a way that at the end of twelve days comparisons had been made between fasting levels, and levels in midmorning, early afternoon, late afternoon, and after supper. Blood analyses showed that variations in hemoglobin, serum protein, chloride, and ascorbic acid were small during these collection periods. It was impossible however to relate the random specimens of urine with the fasting level, whether the values were expressed as concentration per 100 ml., as concentration corrected to a specific gravity of 1.010, or as excretion per hour. The most useful urine analysis of vitamins to reflect changes in dietary status is therefore on the fasting specimen.

The investigators drew three general conclusions from their studies on the value of blood and urine analysis in nutrition surveys: (1) preference should be given to obtaining fasting specimens rather than to conducting load tests, (2) blood specimens give comparable results regardless of the time of the day, and (3) random specimens of urine are of little value and share little of the value afforded by analysis of fasting levels.

PARA-AMINOBENZOIC ACID AND RICKETTSIAE INFECTIONS

The "sulfa" drugs, penicillin and other forms of chemotherapy, which have been so strikingly successful in bacterial infections, have been, with few exceptions, ineffective in viral and rickettsial diseases. The reason for this rather sharp difference in susceptibility is not known. However, since these resistant types of infective agents, in contrast to bacteria, are obligate, intracellular parasites, it has been generally assumed that in some way the environment of the cell prevents the effectiveness of these drugs. The spectacular successes of the chemotherapeutic approach to the treatment of disease, the simplicity of treatment, and promptness of results when an effec-

tive agent is found, make it certain that continued vigorous efforts will be made to find chemotherapeutic agents for the intracellular parasites. Although it is possible that such chemotherapeutic drugs will become known by accident or through a process of trial of all classes of available compounds, it seems likely that such trials will be guided by some rationale based upon our knowledge of the chemical and metabolic nature of the infective agent and the body cells.

An approach of this kind has already been made and with some success in several of the rickettsial diseases. D. Greiff, H. Pinkerton, and V. Moragues (*J. Exp. Med.* **80,** *561* (*1944*)) have pointed out that rickettsial growths are often associated with low rates of metabolic activity in the host cells. In contrast, there is evidence that many viruses grow most favorably in cells which show a very active metabolism. This may prove to be a useful distinction even though it seems likely that this simple rule will not apply to all viruses and rickettsiae. The important concept, however, is that intracellular infective agents appear to have predilections for particular cells in particular metabolic states. This suggests the possibility that small changes in the metabolic state within the cells may create an unfavorable environment for the growth and multiplication of potential invaders and serve as a rationale for the search for chemotherapeutic drugs.

Judging from their introductory remarks, it was reasoning along these lines that led Greiff, Pinkerton, and Moragues to test the effects of para-aminobenzoic acid, among other compounds, as a therapeutic agent against murine typhus in mice. A striking degree of protection was produced against this rickettsial disease by feeding mice a diet of dog chow to which had been added 3 per cent para-aminobenzoic acid (PAB). For example, a survival rate approaching 100 per cent occurred in the treated group of animals as compared with a survival rate of zero in the untreated control group. These observations confirm earlier almost identical experiments of J. C. Snyder, J. Maier, and C. R. Anderson (*Report to Division of Medical Sciences, National Research Council, Dec. 26, 1942*). The beneficial effects of PAB on the clinical course of louse-borne typhus in man were established by the United States of America Typhus Commission Unit in Egypt at about this same time.

That the chemotherapeutic effects of PAB are not confined to typhus is indicated by a recent report of L. Anigstein and M. N. Bader (*Science* **101,** *591* (*1945*)). When guinea pigs were fed 2 per cent PAB in their diet a strikingly favorable change occurred in their response to Rocky Mountain spotted fever. The death rate in control animals with this rickettsial disease is 100 per cent. Out of 12 animals receiving the PAB preceding the injection by twenty-four hours, 7 remained completely afebrile while 5 showed only mild reactions, and only 3 guinea pigs died. In another experiment 80 per cent of the animals remained symptom-free. Furthermore, as many as 30 per cent of the animals remained free of symptoms even when the drug was given as late as seventy-two hours after the inoculation. It was observed that some of the animals which remained afebrile nevertheless showed pathologic lesions in the spleen. When extracts of such spleens were injected into normal controls, typical spotted fever developed. A further interesting observation was noted. When the remaining afebrile animals were reinoculated with spotted fever rickettsiae, they proved to be completely immune. The authors concluded that the afebrile guinea pigs had an unapparent Rocky Mountain spotted fever infection when receiving PAB which resulted in the development of immunity, and speculate that the drug suppresses the virulence of the rickettsiae rather than destroys them.

These encouraging results raise the hope

that chemotherapeutic drugs will be found which are as effective against intracellular parasites as present drugs are against bacterial infection.

RICKETS AND TOOTH DEVELOPMENT

The relation of experimental rickets to the development and calcification of enamel, dentin, cementum, and alveolar bone has been studied under a variety of conditions by several investigators. One of the early experimental studies in the field of nutrition was the production of rickets in dogs. During the preliminary observations of the vital role of the antirachitic vitamin, now known as vitamin D, as a controlling agent in bone calcification, it became apparent that definite defects occurred in the development and calcification of the teeth of rachitic dogs (M. Mellanby, *Lancet* **II,** *767* (*1918*)). The rachitic diet consisted largely of cooked cereals with additions of separated milk powder, raw lean meat, orange juice, brewers' yeast, and sodium chloride. When the teeth which were in the process of development during the vitamin D deficiency erupted, it was found that they were irregular in position with obvious defects in the enamel ranging from a slight hypoplasia and staining to a complete absence of the enamel over large areas of the teeth. The dentin of these teeth had a wide, irregular developmental zone with many atypical, isolated centers of calcification (calcospherites), which never fused to produce normally calcified dentin. The cementum likewise was irregular and poorly calcified. M. Mellanby (*Med. Res. Council, Special Report Ser. no.* **140** (*1929*)) presented an excellent description of the gross and minute changes which occurred in the developing dental structures during rickets. The resumption of normal development and calcification occurred almost immediately if the rachitic diet was supplemented with a source of vitamin D.

Rickets in children has been believed to be associated with the production of enamel hypoplasia. However in a survey of the case histories of 60 children who had hypoplastic defects, B. G. Sarnat and I. Schour (*J. Am. Dental Assn.* **28,** *1989* (*1941*); **29,** *67* (*1942*)) found signs of rickets in only 10 of the children. Even in some of these 10 children the hypoplasia had occurred previous to the indications of rickets. On the basis of this survey it was apparent that rickets was not the sole cause of enamel hypoplasia and possibly not one of the most important causes.

A further example of our incomplete knowledge of the processes of enamel and dentin development has been presented by J. P. Weinmann and I. Schour (*Am. J. Path.* **21,** *821* (*1945*)) in a study on the effect of a rachitogenic diet, high in calcium, low in phosphorus, and deficient in vitamin D, on the developing dental tissues of the white rat. Even after as long as fifty-six days on the rachitogenic diet, the enamel of the incisor showed no disturbance in its formation, calcification, and pigmentation. In addition, no evidence of enamel hypoplasia was observed. The enamel organ was normal in the basal half of the incisor but showed cystic degeneration in the incisal half. The regions of enamel formed in the molar roots during the experimental period showed no significant changes. The rate of dentin formation, however, was retarded while the calcification of dentin was not only retarded but was also imperfect. The width of predentin increased greatly and the newly formed regions of dentin had a definitely interglobular calcification. It was of interest to note that the enamel-covered dentin was more severely affected than the cementum-covered dentin, which is just the reverse of the situation in the vitamin A deficient rat. The formation of cementum

in the vitamin D deficient rats was normal but its calcification was defective.

J. P. Weinmann and I. Schour (*Am. J. Path.* **21,** *833 (1945)*) described the changes of the alveolar bone which occurred in the previously mentioned rachitic rats. The apposition of alveolar bone appeared to proceed at a normal rate but remained as uncalcified osteoid tissue which failed to undergo the normal process of resorption. This imbalance between apposition and resorption resulted in a distortion of the growth pattern of the alveolar bone. The periodontal space was frequently reduced by the excessive accumulation of osteoid tissue which led to the compression of the periodontal membrane and its blood vessels. Hyaline degeneration of the connective tissue occurred with occasional areas of complete obliteration of the periodontal membrane. Histologic evidence indicated that the rate of eruption of the molars and incisors had been greatly reduced. This disturbance of eruption appeared to be secondary to the retardation of bone growth at the mandibular condyle.

The studies of Weinmann and Schour on the effect of a rachitogenic diet, low in phosphorus and deficient in vitamin D, on the dental tissues of the white rat provide only a partial answer to the relation of rickets and tooth development. The lack of enamel hypoplasia in the white rat on the described dietary regimen is in direct contrast to the extreme enamel hypoplasia observed in the rachitic dog. Part of this difference may have been due to the much higher phosphorus content of Mellanby's diet. Undoubtedly there is also a species difference to be considered especially since it is not necessary to disturb the calcium-phosphorus ratio of a vitamin D deficient ration in order to produce rickets in the dog. The cystic degeneration of the enamel organ in the incisal half of the incisor described by Weinmann and Schour presents an interesting point for speculation as to whether this degeneration might have progressed to the basal half of the enamel organ where enamel development is actively occurring if the rachitic process had been more severe or more prolonged. If the basal half of the enamel organ had become thus involved, it is feasible to expect that there might have been some effect on enamel development. There probably can be no complete answer given on the relation of rickets to enamel hypoplasia in man until the pathologic effects of rickets of varying types and degrees on the developing enamel are more thoroughly demonstrated.

NUTRITION IN ARMY TRAINING CAMPS

Detailed surveys of food consumption in army training camps have been made during two wars by J. R. Murlin and F. M. Hildebrandt (*Am. J. Physiol.* **49,** *531 (1919)*) and P. E. Howe and G. H. Berryman (*Ibid.* **144,** *588 (1945)*). As a result much information of high statistical value is available regarding the food intake of active young adult males, representing a large cross section of our population.

In World War I, Murlin and Hildebrandt determined the average food consumption of soldiers in four hundred and twenty-seven messes in sixty-seven camps. Information obtained included the weight of each of the three main food types supplied per day, together with the cost and the amount wasted. The average daily consumption of nutrients was 122 g. protein, 123 g. fat, and 485 g. carbohydrate, which furnished 3633 calories per man at a cost of 44 cents. An additional 266 calories was wasted. The average value varied only slightly among men of different organizations after an initial period of about five weeks, during which time the caloric intake rose from about

2700 to 3600 calories. Food consumption showed some seasonal variations, being lowest in May (3514 calories) and rising in December to 4145 calories.

Estimates of food obtained outside mess halls, e.g., at canteens, were difficult to obtain accurately, for obvious reasons. The best estimate was given as 365 calories per man at a cost of 22 cents, which gives a total of approximately 4000 calories per day. This was considered ample during the training period, as was evidenced by an average gain in weight of 6.6 pounds per man during the course of four or five months training.

The survey made during 1941 to 1943 by Howe and Berryman confirms to a remarkable degree the earlier findings and extends it to include estimates of mineral and vitamin intake by the men of fifty posts in four hundred and fifty-five messes. Averages obtained indicated a daily intake of 3694 calories per man, with occupational extremes ranging from 3132 calories in induction stations to 4135 calories for demolition squads, and seasonal extremes extending from 3570 calories in the spring to 3960 calories in the late fall. Food from canteens was thought to contribute 400 additional calories per day. Waste amounted to 0.39 pound per day, compared with the World War I value of 0.38.

The average values were distributed as follows among the food types: protein 125 g., fat 178 g., and carbohydrate 408 g. The nutritional pattern had thus changed somewhat in that more fats were added to the diets in World War II, at the expense of carbohydrate. The higher fat contribution to the diet (about 1600 calories) represents well over one third of the total caloric intake, an amount which is in line with the current recommendations of the Food and Nutrition Board of the National Research Council (*Nutrition Reviews* **3,** *287 (1945)*).

Requirements for accessory food factors appear to have been well met in the training camps during the war just ended. Calcium levels averaged 923 mg. per day, phosphorus 1885 mg., and iron 22 mg. The vitamin A supplied to the diet was 11,900 I.U., while the levels of thiamine, riboflavin, niacin, and ascorbic acid were estimated at 2.1, 2.3, 22.5, and 74 mg. respectively. These values meet the Food and Nutrition Board requirements generally for a high calory diet, although the supply of B-vitamins on the army regimen is slightly less than the average civilian intake when both are calculated on a 2500 calory basis. When this is done, the army diet is found to yield 1.3 mg. thiamine, 1.4 mg. riboflavin, and 14 mg. niacin, compared with 1.3, 1.6, and 17 mg. respectively in an average American diet which was supplemented with enriched bread and flour (R. L. Lane, E. Johnson, and R. R. Williams, *J. Nutrition* **23,** *613 (1942)*; V. H. Cheldelin and R. R. Williams, *Ibid.* **26,** *417 (1943)*).

A feature of the army diets is the relatively low protein content when compared with diets of other laboring groups such as the American farmer (150 g. protein in a 4150 calory diet) or the Finnish peasant (136 g. protein in a 3500 calory diet). However, the level of approximately 125 g. is far in excess of the 70 g. minimum recommended by the Food and Nutrition Board. It would appear that the diet in the training camps satisfies the known nutritional requirements with a good margin of safety.

ANEMIA AND GRANULOCYTOPENIA IN RELATION TO PANTOTHENIC ACID AND "FOLIC ACID" DEFICIENCIES

The understanding of agranulocytosis or the less acute granulocytopenia has always been of particular interest to the clinician because these disturbances develop not

infrequently in patients treated with various chemotherapeutic drugs. In 1942 A. D. Welch, P. A. Mattis, and A. R. Latven (*J. Pharmacol. Exper. Therap.* **75,** *231 (1942)*) and S. S. Spicer, F. S. Daft, W. H. Sebrell, and L. L. Ashburn (*Pub. Health Reports* **57,** *1559 (1942)*) described the appearance of serious growth disturbances and blood dyscrasias (agranulocytosis, bone marrow hypoplasia) in rats given sulfaguanidine and sulfasuxidine in purified diets. It was found that the blood dyscrasia due to feeding of purified diets containing sulfa drugs could be successfully treated with certain liver extracts known to be rich in "folic acid" (Spicer *et al., loc. cit.*; A. E. Axelrod, P. Gross, M. D. Bosse, and K. F. Swingle, *J. Biol. Chem.* **148,** *721 (1943)*); B. Ransone and C. A. Elvehjem, *Ibid.* **151,** *109 (1943)*); A. Kornberg, F. S. Daft, and W. H. Sebrell, *Science* **98,** *20 (1943)*).

The isolation of "folic acid" (L. casei factor "Bc") by J. J. Pfiffner *et al.* (*Science* **97,** *404 (1943)*) and E. L. R. Stokstad (*J. Biol. Chem.* **149,** *573 (1943)*) made it possible to study the role of "folic acid" in these types of blood dyscrasias. F. S. Daft and W. H. Sebrell (*Pub. Health Reports* **58,** *1542 (1943)*) showed clearly that blood dyscrasia induced by sulfa drugs in purified diets could be treated successfully with crystalline "folic acid." A. Kornberg, F. S. Daft, and W. H. Sebrell (*Proc. Soc. Exp. Biol. Med.* **58,** *46 (1945)*) also found that dietary agranulocytosis developed in a small percentage of rats on purified diets even without added sulfa drugs. Experimental agranulocytosis produced in this manner also responded favorably to addition of crystalline "folic acid." It seemed evident that although agranulocytosis can be produced by dietary means only, the incidence can be greatly increased by including sulfa drugs in the diet.

F. S. Daft, A. Kornberg, L. L. Ashburn, and W. H. Sebrell (*Pub. Health Reports* **60,** *1201 (1945)*) now report that severe anemia, granulocytopenia, and bone marrow hypoplasia can also be developed in a high percentage of rats fed a purified diet low in pantothenic acid, while control rats receiving adequate amounts of pantothenic acid show almost no deficiency signs. However, despite this manifest effectiveness of pantothenic acid in preventing these blood dyscrasias in the control animals, the vitamin was not so effective in alleviating the blood and bone marrow changes once they developed.

Groups of rats were placed on a diet consisting of leached and alcohol extracted casein, 18 per cent; Crisco, 8 per cent; salt mixture, 4 per cent; dextrose; iron and copper salts; all the vitamins of the B-group with the exception of pantothenic acid; vitamins A, D, and K; and alpha-tocopherol. The control animals were fed the same diet plus 200 micrograms of pantothenic acid daily.

Hemoglobin and hematocrit determinations as well as polymorphonuclear granulocyte counts were made at various times. The term granulocytopenia was used whenever the granulocyte count fell below 400 cells per cubic millimeter. A hematocrit of 39 per cent or lower was used as the standard for anemia.

On this dietary regimen some animals developed an anemia, some a granulocytopenia, others showed both changes, while some showed no changes at all. In one example given, only 20 per cent of the rats escaped the development of some form of a blood dyscrasia. The authors mention, however, that the various dyscrasias occur in varying proportions and severity from experiment to experiment. The most severe anemia occurred in animals which were also granulocytopenic. In some rats, the onset of blood dyscrasias, particularly the anemia, was extremely rapid and was followed closely by a fatal termination of the disease. The bone marrow of the rats with both anemia and granulocytopenia showed distinct or

marked hypoplasia with varying degrees of congestion.

Groups of animals showing each type of blood change were treated with pantothenic acid, "folic acid," or both, and observed over a period of days for response. The detailed tables which are given in the paper show that doses of 200 to 5000 micrograms daily of pantothenic acid were used alone or in conjunction with 25 to 100 micrograms of the "L. casei factor." The therapeutic observations were made difficult by the fulminating character of the disease and therefore prevented the clearcut results for which the authors had hoped. Nevertheless, certain conclusions were possible.

The granulocytopenia when unaccompanied by anemia appeared in most cases to be the result of an L. casei factor deficiency which, for reasons unknown, developed more easily in the absence of pantothenic acid. Although the anemia improved somewhat by the treatment with pantothenic acid, the response was slow and incomplete. It seemed probable that anemia, like the granulocytopenia, was not the direct result of pantothenic acid deficiency but rather developed indirectly due to the deficiency of an unknown factor which became apparent in the absence of pantothenic acid.

"Folic acid" alone did not affect the anemia, and pantothenic acid alone did not affect the granulocytopenia. The anemia responded reasonably well to the combined treatment of pantothenic acid and "folic acid." It would seem therefore at least a possibility that the anemia was due to the simultaneous deficiency of these two factors rather than to any unknown factor. However, the work has not progressed sufficiently to reach a decision on this point.

These preliminary observations are of importance in emphasizing the possibilities of one deficiency creating conditions which lead to a deficiency of other factors, and thus leading to the development of a rather complex pathologic picture. The observations of L. D. Wright and A. D. Welch (*J. Nutrition* **27,** *55 (1944)*) are of interest in a discussion concerning this relationship. They found that feeding rats a highly purified diet containing sulfasuxidine caused a marked reduction in the hepatic stores of "folic acid," biotin, and pantothenic acid, whereas the storage of the other vitamins of the B-group remained unchanged. The drop in the hepatic store of pantothenic acid was so much the more remarkable because the purified diet contained amounts of pantothenic acid which under ordinary conditions would be amply adequate. Increasing the dietary intake of pantothenic acid or giving the vitamin parenterally did not cause a renewal of growth, failed to modify the signs of pantothenic acid deficiency, and left unaffected the severely reduced amount of pantothenic acid in the liver. Administration of crystalline biotin plus a concentrate of "folic acid" caused a prompt recovery from the signs of pantothenic acid deficiency and a restoration of the pantothenic acid content of the liver to normal.

Wright and Welch discuss the possibility of "folic acid" and biotin playing a role in the bacterial synthesis of pantothenic acid, but venture also the possibility that "folic acid" and biotin may be essential for the proper storage or utilization of pantothenic acid in the tissues.

SOLUBILITY AND BIOLOGIC ACTIVITY OF RIBOFLAVIN DERIVATIVES

Since riboflavin has such a low solubility in water (12 mg. per 100 ml. water at 27.5°C.) attempts have been made to prepare more soluble derivatives which would retain the

biologic potency of the flavin itself. The riboflavin molecule offers two possibilities for relatively simple substitution: in the ring at the imino nitrogen, or in the ribose chain. R. Kuhn and H. Rudy (*Ber. d. deutsch. chem. Gesellsch.* **69,** *2557 (1936)*) prepared some ring-substituted derivatives and found that these compounds did not fluoresce like riboflavin, and were biologically inactive.

Substitution in the ribose chain can give rise to esters or acetals. Among the esters, R. Kuhn and H. Rudy (*Ber. d. deutsch. chem. Gesellsch.* **68,** *383 (1935)*), R. Kuhn, H. Rudy, and F. Weygand (*Ibid.* **69,** *1543 (1936)*), and P. Karrer, P. Frei, and H. Meerwein (*Helv. chem. acta* **20,** *79 (1937)*) have described the phosphate; R. Kuhn and T. Wagner-Jauregg (*Ber. d. deutsch. chem. Gesellsch.* **66,** *1577 (1933)*) have described the acetates; and D. V. Frost (*J. Biol. Chem.* **145,** *693 (1942)*) has described the borate and the tetrabenzoate. The phosphates and acetates were shown to be biologically active, but no tests were reported for the borate or tetrabenzoate. R. Kuhn, H. Rudy, and F. Weygand (*Ber. d. deutsch. chem. Gesellsch.* **68,** *625 (1935)*) prepared two acetals, the monoacetone and diacetone derivatives of riboflavin, and R. Kuhn (*Z. angew. Chem.* **49,** *6 (1936)*) claimed that the diacetone compound is active, without presenting any data.

M. F. Furter, G. J. Haas, and S. H. Rubin (*J. Biol. Chem.* **160,** *293 (1945)*) have extended the search for soluble, active derivatives. Some additional compounds were prepared and assayed fluorometrically, by the microbiologic method of Snell and Strong, and by the rat growth method of H. R. Street (*J. Nutrition* **22,** *399 (1941)*). Two new acetals were prepared by condensing riboflavin with levulinic acid and with chloral. The two condensation products were fluorescent but were biologically inactive both for Lactobacillus casei and for the rat. This casts doubt upon the earlier claim that the diacetone derivative is biologically active.

Four succinates were also prepared by Furter, Haas, and Rubin by treatment of riboflavin with increasing proportions of succinic anhydride in dry pyridine. The solubility of the succinates increases with increasing substitution of the riboflavin molecule.

Fluorometric measurement of solutions of the four succinates showed that the riboflavin present in the esters fluoresced to the same extent as if it were present in the free form. The rat assay showed the monosuccinate to be fully active (and eight times more soluble in water), the disuccinate to have 65 per cent, the trisuccinate 21 per cent, and the tetrasuccinate none of the theoretical activity due to the riboflavin content. For L. casei the observed activities of the succinates were lower than for the rat. The monosuccinate and disuccinate were 57 and 18 per cent respectively as active as the theoretical value due to their riboflavin content, and both the trisuccinate and tetrasuccinate were completely inactive.

The inactivity of the tetrasuccinate for the rat may be contrasted with the observation by P. György, R. Kuhn, and T. Wagner-Jauregg (*Z. f. physiol. Chem.* **223,** *241 (1934)*) that the tetra-acetate is active. Furter, Haas, and Rubin concluded from assay experiments that the rat could utilize these esters more fully than L. casei. In this characteristic the succinates resemble four flavins of the group examined by E. E. Snell and F. M. Strong (*Enzymologia* **6,** *186 (1939)*), which were classified as "active for the rat" but which showed only partial activity for L. casei.

NOTES

Letters to the Editor

Dear Sir:

The ultimate proof of metabolic transformations that is frequently supplied by tracer studies may lead the enthusiastic investigator to forget the fundamental background and the original formulation of a hypothesis. In many cases the application of the tracer method merely furnishes confirmatory evidence in support of observations that have been well established by the more usual types of biologic experimentation. Recorded progress in the field of tracer studies is marked by several "rediscoveries" of well-known metabolic phenomena.

The purpose of this letter is to point out one such instance that occurs in a review of carbohydrate metabolism in diabetes (*Nutrition Reviews* **3,** *213* (*1945*)) in which the excellent paper of D. W. Stetten and G. E. Boxer (*J. Biol. Chem.* **156,** *271* (*1944*)) on metabolic defects in alloxan diabetes is discussed. The reviewer in commenting on the observation of Stetten and Boxer that there is an impairment of fatty acid synthesis in the diabetic animal, describes this as a new metabolic finding in diabetes. No reference is made by the reviewer to the papers of Drury and his associates (D. R. Drury, *Am. J. Physiol.* **131,** *536* (*1940*), F. Pauls and D. R. Drury, *J. Biol. Chem.* **145,** *481* (*1942*)) in which extensive evidence of the action of insulin in accelerating the transformation of glucose to fat has been presented. It is true that Stetten and Boxer also fail to mention this earlier work. However, the reviewer states: "This new metabolic finding in diabetes invites a discussion of other recent interpretations of the action of insulin and the mechanism of fat synthesis." In any such discussion the observations of Drury and his associates would indeed be pertinent.

Richard H. Barnes,
Department of Biochemical Research,
Sharp and Dohme, Inc.,
Glenolden, Pennsylvania.

Dear Sir:

It is unfortunate that the comments on "Thiamine Requirements at High Temperatures" (*Nutrition Reviews* **3,** *187* (*1945*)) should have been prepared entirely without regard to two facts having very important bearings on the subject, namely: (1) That two to three weeks are required for metabolic adaptation to heat, a point I have emphasized repeatedly and dealt with specifically in a recent article (*Am. J. Trop. Med.* **25,** *59* (*1945*)). On this account, the work of O. L. Kline *et al.* should be considered only in its bearing on brief heat exposure and thus should not be used in efforts to negative our findings. In no case did they present data obtained after the animals had been given time to make the metabolic adjustments to environmental temperature upon which their differences in nutritional requirements are based. (2) That all my claims regarding a heightened thiamine requirement in tropical heat have dealt with dietary concentration (milligrams per kilogram of diet) and not with micrograms per rat per day. I purposely used this approach to the problem because I wished to know whether diets adequate by temperate zone standards might not be quite inadequate when fed to tropical residents, simply because a lessened appetite in the heat leads to a lowered intake of the vitamin-containing foods. Our studies have shown that higher dietary concentrations of thiamine (milligrams per kilogram of diet) are needed by animals

adapted to tropical warmth. It is not true, as stated in the later review dealt with in the next paragraph, that these findings have not been confirmed elsewhere, for H. P. Sarett and W. A. Perlzweig (*J. Nutrition* **26,** *611 (1943)*) also found that diets with thiamine percentage adequate for optimal growth at 75°F. became deficient when fed to rats adapted to 91°F.

It is also unfortunate that the comments (*Nutrition Reviews* **3,** *317 (1945)*) on my paper "Metabolic Acclimatization to Tropical Heat," should have been prepared without greater familiarity with the pertinent literature in the field. My statements were not "based upon evidence derived largely or exclusively from experiments on small animals" but upon a previous careful survey (*Am. J. Hygiene* **29,** *147 (1939)*) of published works in this field, most of which dealt with human responses to climatic or seasonal temperature differences. Perusal of these works would have prevented the reviewer from making the statement: "Such an adaptation has never been demonstrated for man, though there is some evidence based on the study of only 2 subjects. . . . Generalizations based upon such scanty data are dangerous." Much more dangerous are uncritical and poorly based statements by reviewers, for they tend to rob an author's words and works of their proper value and significance.

Clarence A. Mills, M. D.
Professor of Experimental Medicine,
University of Cincinnati.

Dear Sir:

Your excellent journal becomes increasingly indispensable for all who are concerned with problems of nutrition. I am sure that many must share with me the belief that Nutrition Reviews could well be taken as a model for equally needed expositions of the developments of other fields.

An outstanding characteristic of the Reviews is the frequent elucidation of basic scientific method and analysis as it emerges in these brief discussions. The attempt to evaluate the significance of various findings often goes beyond the limitations of the immediately factual technicalities and thereby reminds us that the scientific method is only the ordered arithmetic of general logic.

In the November issue of the Reviews there are two articles which seem to be timely and which bear on my foregoing remarks. Dr. Muench's discussion of the role of the statistician is excellent and should be taken to heart by all of us. The discussion of "Nutritional Requirements" (p. 323) emphasizes the virtue of reasonable sanity in a controversial area. It is, as stated, "unfortunate that the Table of Allowances has been misinterpreted by certain groups."

I am sure that the Nutrition Foundation and the Editorial Staff must be gratified at the success of Nutrition Reviews. May it continue to prosper.

Ancel Keys,
Director, Laboratory of
Physiological Hygiene,
University of Minnesota,
Minneapolis, Minnesota.

Nutrient Allowances for Domestic Animals

The National Research Council, Committee on Animal Nutrition, 2101 Constitution Avenue, N.W., Washington, D. C., has prepared an interesting series of bulletins on "Recommended Nutrient Allowances for Domestic Animals." The series (numbered 1 to 5 respectively) refer to the allowances for poultry, swine, dairy cattle, beef cattle, and sheep. Price: 25¢ each.

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